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13. ABSTRACT (Maximum 200 Words)

The purpose of this research is to discover protein markers associated with the early development of breast cancer. One of the earliest common changes in this process occurs when some breast epithelial cells begin to grow and proliferate independently of estradiol. Normal breast epithelium requires this hormone for growth and proliferation. Dr. Robert Clarke of Georgetown University has provided us with two breast epithelial cell lines: MCF7 cells, whose proliferation is dependent on estradiol; and LCC1 cells, a cell line derived from MCF7 whose proliferation is independent of estradiol. We have begun applying proteomics techniques (two dimensional electrophoresis and mass spectrometry) in order to characterize broadly the patterns of protein expression in these two cell lines both in the presence and in the absence of estradiol. We have confirmed Dr. Clarke's previous findings that the patterns of protein expression of the LCC1 cells are not much affected by estradiol and more closely resemble the those patterns seen in the estradiol-stimulated MCF7 cells than they do the unstimulated MCF7 cells. We have begun to identify the estradiol-induced protein changes by mass spectrometry and have implemented methods for subcellular fractionation in order to increase the depth and breadth of our discovery efforts.

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INTRODUCTION

The purpose of this research is to discover protein markers associated with the early development of breast cancer. One of the earliest common changes in this process occurs when some breast epithelial cells begin to grow and proliferate independently of estradiol. Normal breast epithelium requires this hormone for growth and proliferation. Dr. Robert Clarke of Georgetown University has provided us with two breast epithelial cell lines: MCF7 cells, whose proliferation is dependent on estradiol; and LCC1 cells, a cell line derived from MCF7 whose proliferation is independent of estradiol. We have begun applying proteomics techniques (two dimensional electrophoresis and mass spectrometry) in order to characterize broadly the patterns of protein expression in these two cell lines both in the presence and in the absence of estradiol. We have confirmed Dr. Clarke's previous findings that the patterns of protein expression of the LCC1 cells are not much affected by estradiol and more closely resemble the those patterns seen in the estradiol-stimulated MCF7 cells than they do the unstimulated MCF7 cells. We have begun to identify the estradiol-induced protein changes by mass spectrometry and have implemented methods for subcellular fractionation in order to increase the depth and breadth of our discovery efforts.

BODY

Representative silver-stained 2D gels of total proteins isolated from MCF7 cells plus or minus 17-β-estradiol (E2) and unstimulated LCC1 cells are shown in Figures 1, 2 and 3, respectively. These represent three of the nearly 160 such gels that have been run during the first year of this work. The overall similarity of the proteins displayed here speaks well of the reproducibility of our techniques. Each gel contains roughly 1200 recognizable protein features, most of which can be mapped from one gel to the others and also which do not change appreciably. Most of our initial time in this study were spent replicating Dr. Clarke's conditions as closely as possible by harvesting the cells 24 hours after addition of E2 to the medium. The effect of E2 on the proteomes of the MCF7 cells was virtually non-existent. That is to say the proteins detected in 2D gels from estrogen-stimulated cells were virtually indistinguishable from those of unstimulated cells. More consistent changes were observed when comparing MCF7 with LCC1 cells. Following this observation we decided to compare the effect of E2 on the growth curves of MCF7 and LCC1 cells. Figure 4B shows that the LCC1 cells grow independently of E2 while Figure 4A confirms the dependence on E2 for growth of MCF7 cells. There is, however, a pronounced 1 day lag between the time of E2 addition and detection of increased growth of MCF7 cells (Fig. 4A). When we increased the time of re-exposure of the MCF7 cells to E2 to 48 or 72 hours, we could observe many significant and reproducible differences in protein expression patterns induced by E2. Following the analysis of many such gel sets using the Phoretix 2D gel analysis software package (Nonlinear Dynamics) we have confirmed Dr. Clarke's observation of nucleophosmin induction by E2 and extended those observations to include a dozen or more protein candidates that are either induced or repressed by the action of E2 on MCF7 cells and which appear constituitively induced or repressed in the LCC1 cells, independent of the action of E2. Several examples of Phoretix analyses illustrating some of these consistent changes are shown in Figures 5 and 6. Figure 5 shows protein features downregulated by E2 while Figure 6 shows examples upregulated proteins. While additional protein changes can be observed in these individual gels, when analyzed across several averaged gels, the additional changes appear insignificant or inconsistent. We have begun systematically identifying the E2-responsive proteins by mass spectrometry. Selected MALDI TOF mass spectra for peptide mass mapping protein identification are shown in Figure 7 and Figure 8

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shows some examples of the results of SEQUEST database searches using MSMS data which are used to confim the MALDI TO MS results.

KEY RESEARCH ACCOMPLISHMENTS

- Established MFC7 and LCC1 breast epithelial cultures in my lab.
- Characterized and confirmed growth characteristics of cell lines
- Established 2D electrophoretic techniques for analyzing cell line proteomes
- Have applied 2D gel analysis software to statistical analysis of proteomes
- Have confirmed and extended Dr. Clarke's observations on E2 induced proteome changes
- Have begun to systematically identify E2 regulated proteins by mass spectrometry
- Have initiated subcellular fractionation to answer questions concerning protein subcellular localization

REPORTABLE OUTCOMES

Not yet applicable

CONCLUSIONS

We have confirmed many of the expected E2-induced protein changes in MCF7 and LCC1 cells and have begun to extend these observations to newly identified protein markers. We are working at identifying several newly recognized protein markers associated with the early development of breast cancer.

REFERENCES

Skaar TC. Prasad SC. Sharareh S. Lippman ME. Brunner N. Clarke R. Two-dimensional gel electrophoresis analyses identify nucleophosmin as an estrogen regulated protein associated with acquired estrogen-independence in human breast cancer cells. Journal of Steroid Biochemistry & Molecular Biology. 67(5-6):391-402, 1998
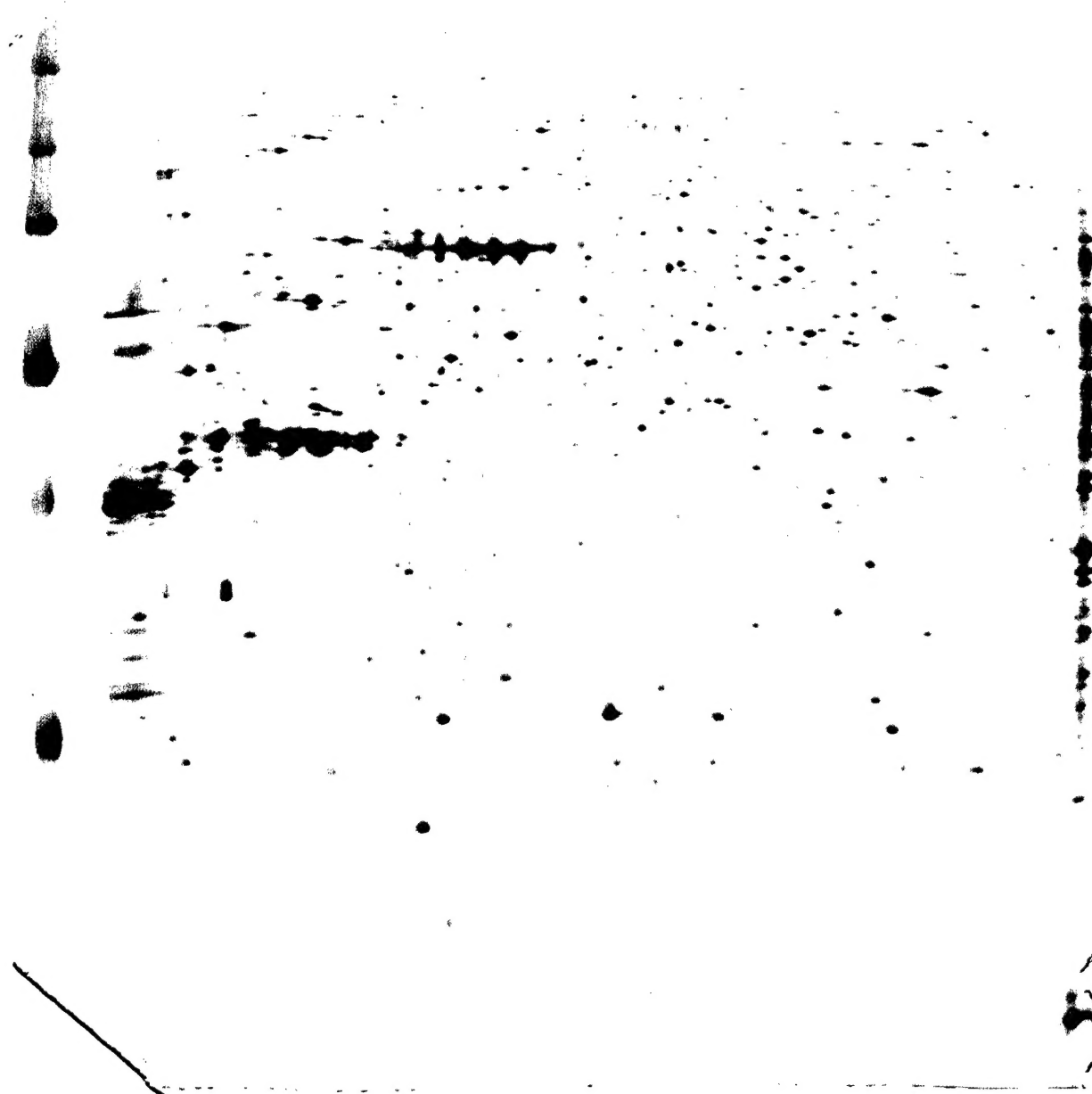
MCF7 –E2

Figure 1. Silver stained 2D gel of proteins from MCF7 cells in the absence of E2. Cells were grown for three days in the presence of bovine calf serum (BCS) and then moved to charcoal stripped calf serum (CCS) for a period of three days. The cells were harvested for protein extraction after an additional two days in IMEM + 5% CCS. The pH range of the immobilized pH gradient strips was 5 to 8 (horizontal axis) and the molecular size (vertical axis) is indicated on the right using BioRad Precision protein standards.
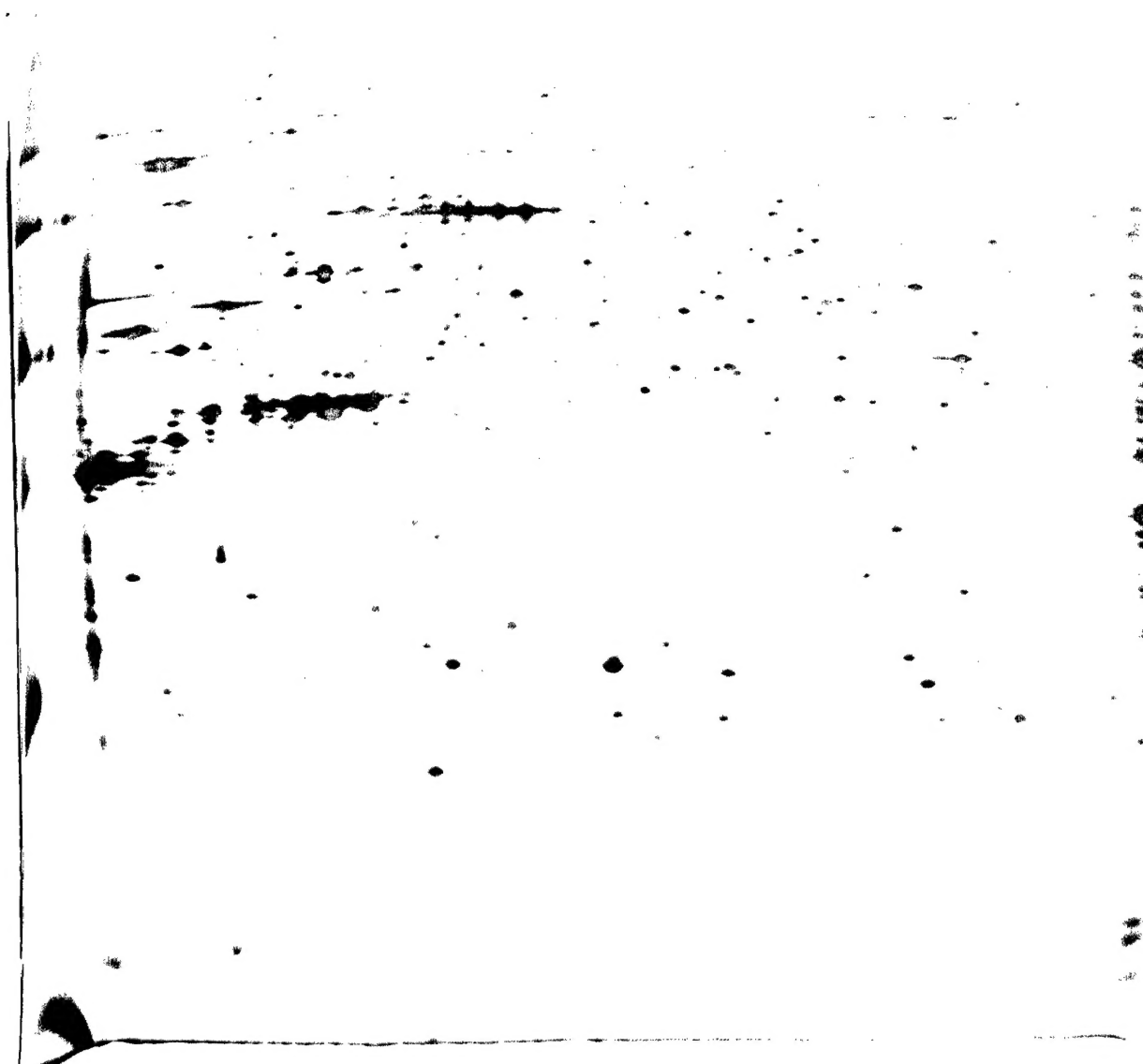
MCF7 +E2

Figure 2. Silver stained 2D gel of proteins from MCF7 cells in the presence of E2. Cells were grown for three days in the presence of bovine calf serum (BCS) and then moved to charcoal stripped calf serum (CCS) for a period of three days. The cells were harvested for protein extraction after an additional two days in IMEM + 5% CCS + 1nM E2. The pH range of the immobilized pH gradient strips was 5 to 8 (horizontal axis) and the molecular size (vertical axis) is indicated on the right using BioRad Precision protein standards.
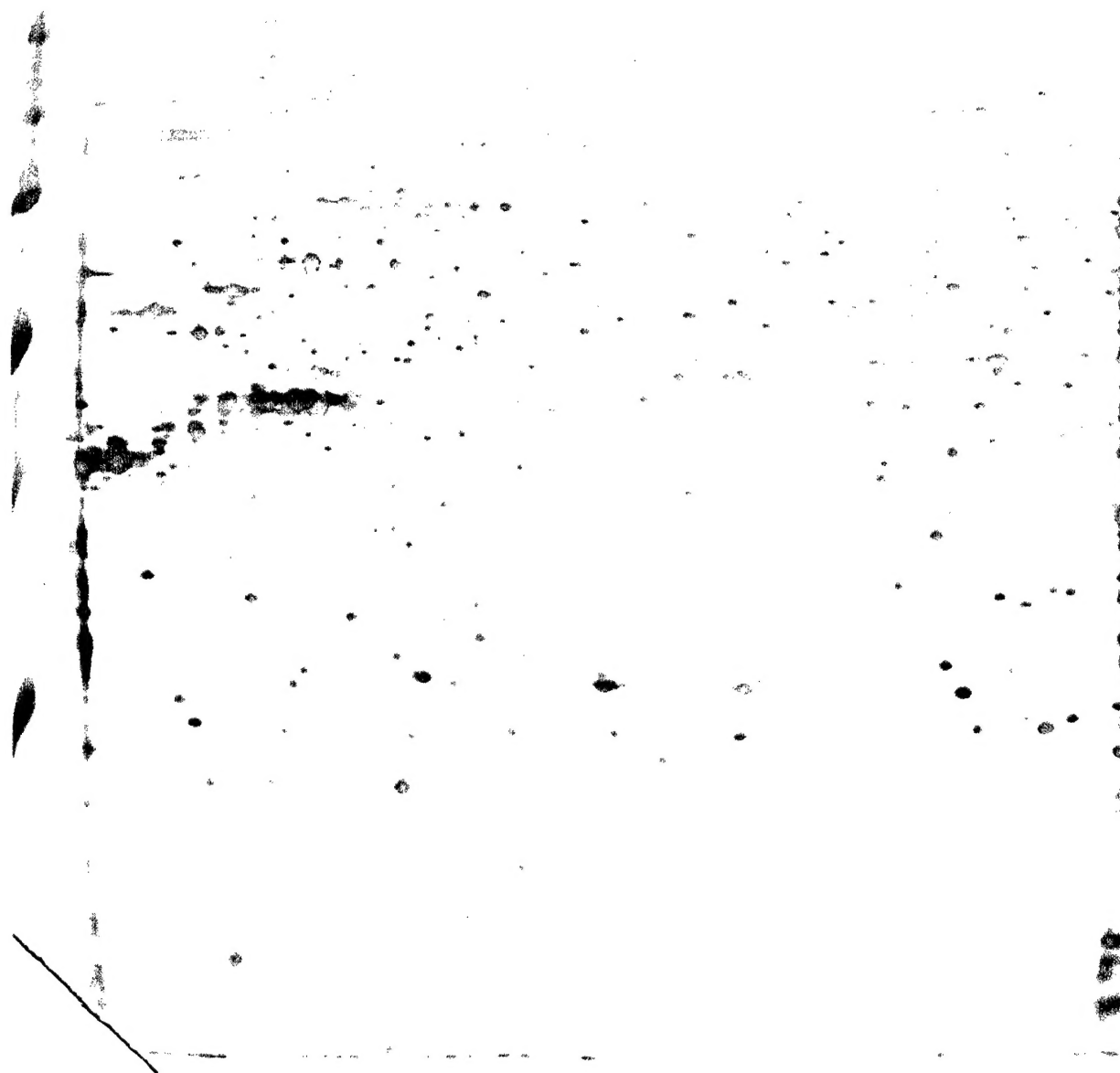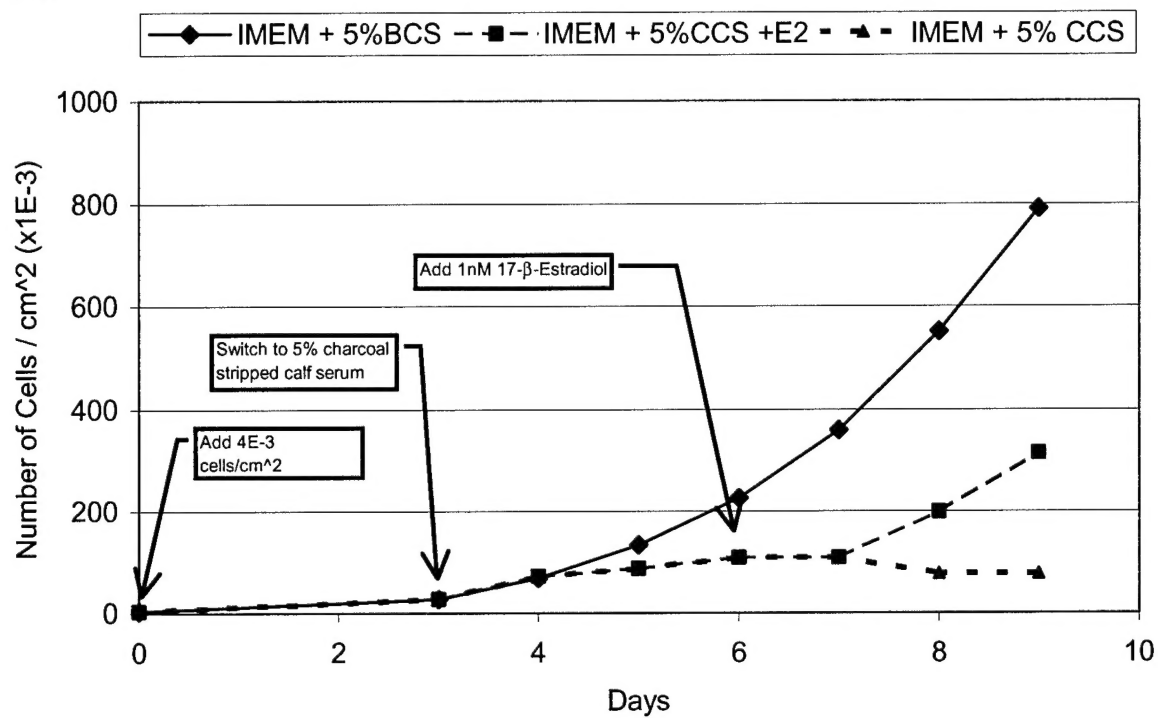
Figure 3. Silver stained 2D gel of proteins from LCC1 cells in the absence of E2. Cells were grown for six days in the presence of charcoal stripped calf serum (CCS). The cells were harvested for protein extraction after an additional two days in IMEM + 5% CCS. The pH range of the immobilized pH gradient strips was 5 to 8 (horizontal axis) and the molecular size (vertical axis) is indicated on the right using BioRad Precision protein standards.

A.

MCF7 Growth Curves: Effect of E2

Legend: —◆— IMEM + 5%BCS – ■ – IMEM + 5%CCS +E2 - ▲ - IMEM + 5% CCS

Y-axis: Number of Cells / cm^2 (x1E-3) — 0, 200, 400, 600, 800, 1000

X-axis: Days — 0, 2, 4, 6, 8, 10

Annotations:
- Add 1nM 17-β-Estradiol
- Switch to 5% charcoal stripped calf serum
- Add 4E-3 cells/cm^2

B.

LCC1 Growth Curves: Effect of E2

Legend: —◆— IMEM + 5%BCS – ■ – IMEM + 5% CCS + E2 - ▲ - MEM + 5% CCS

Y-axis: Number of Cells / cm^2 (*1E-3) — 0, 200, 400, 600, 800, 1000

X-axis: Days — 0, 2, 4, 6, 8, 10

Annotations:
- Add 1nM 17-β-Estradiol
- Switch to 5% charcoal stripped calf serum
- Add 4E-3 cells/cm^2
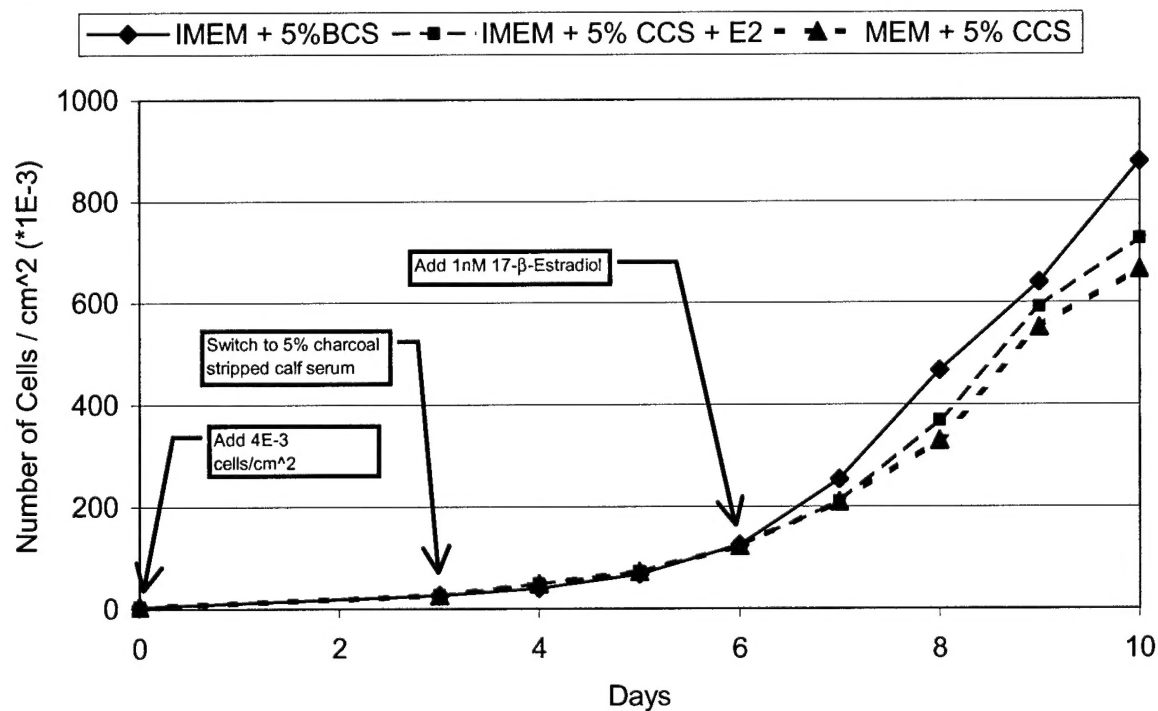
Figure 4. E2 effects on growth of MCF7 and LCC1 cells

A.   MCF7 –E2   B.   MCF7 +E2

GST-Mu1

Thioredoxin
peroxidase

C.

Keratin 8

D.

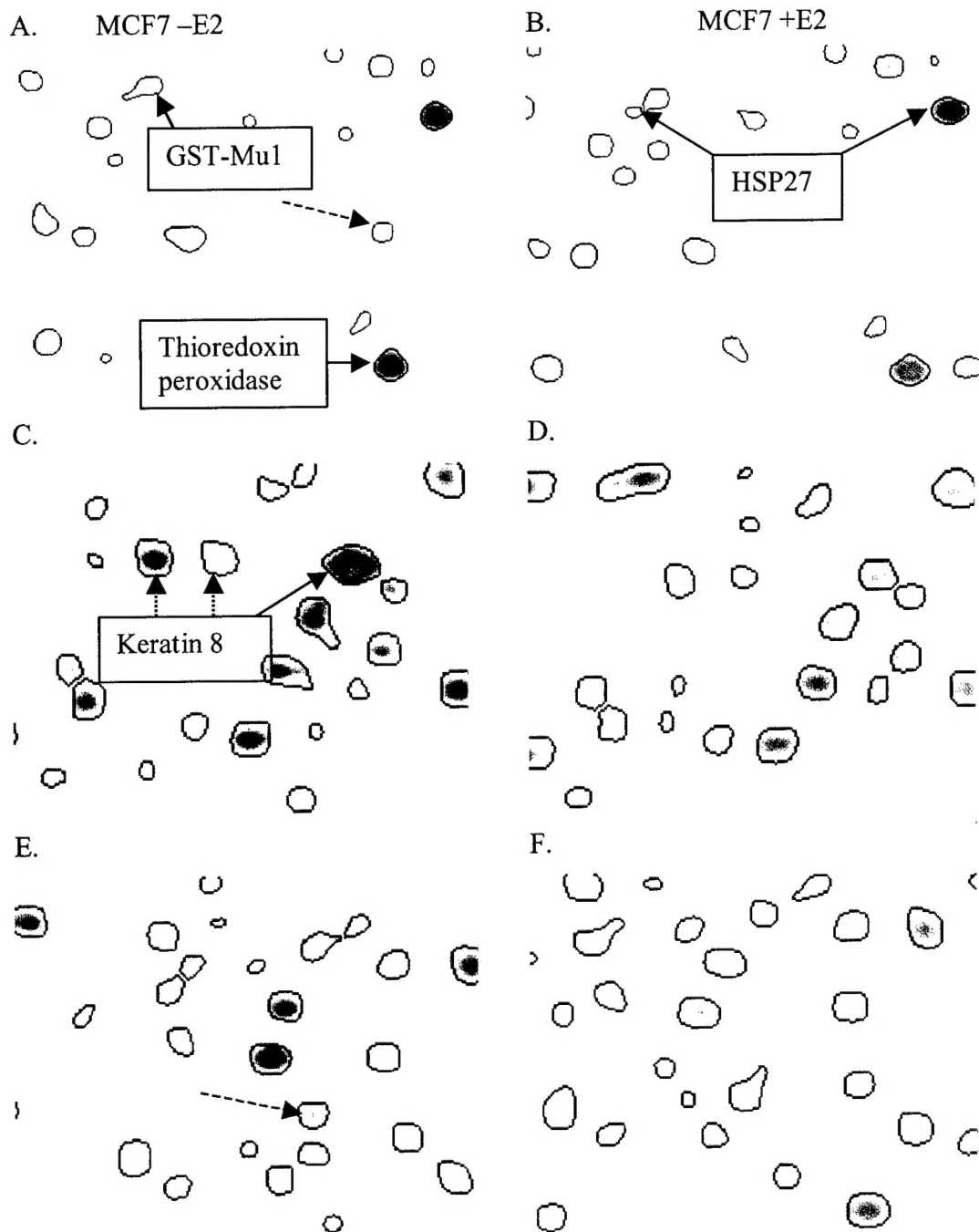HSP27

E.   F.

Fig 5. Estradiol downregulated protein features: Phoretix analyzed gel images indicating consensus of protein changes observed in several gel comparisons. Downregulated protens are indicated by a dashed line. Other known proteins identified by mass spectrometry are indicated by solid lines. Phosphorylated forms of Hsp27 shown in B are upregulated by the action of E2.

Figure 6. Estradiol upregulated protein features.
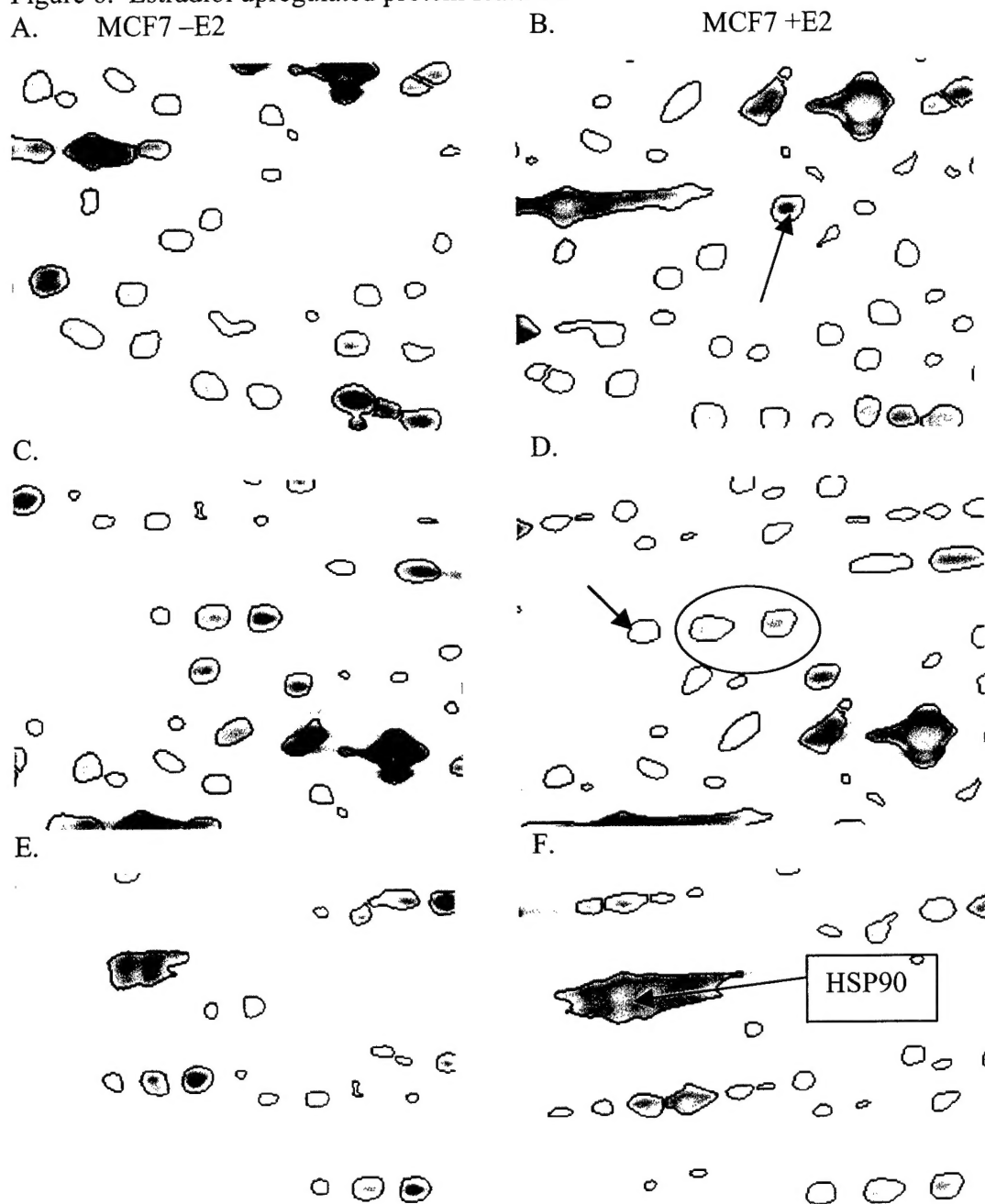A.   MCF7 –E2   B.   MCF7 +E2

Fig 6. Estradiol upregulated protein features: Phoretix analyzed gel images indicating consensus of protein changes observed in several gel comparisons. Upregulated protens are indicated by the arrows. Mass spectrometry data show that the two protein features circled in D represent different modified forms of the upregulated one. The identity of these and the protein in B await additional confirmation by mass spectrometry. The upregulated 90 kDa heat shock protein in F comprises a spot family of four pairs.
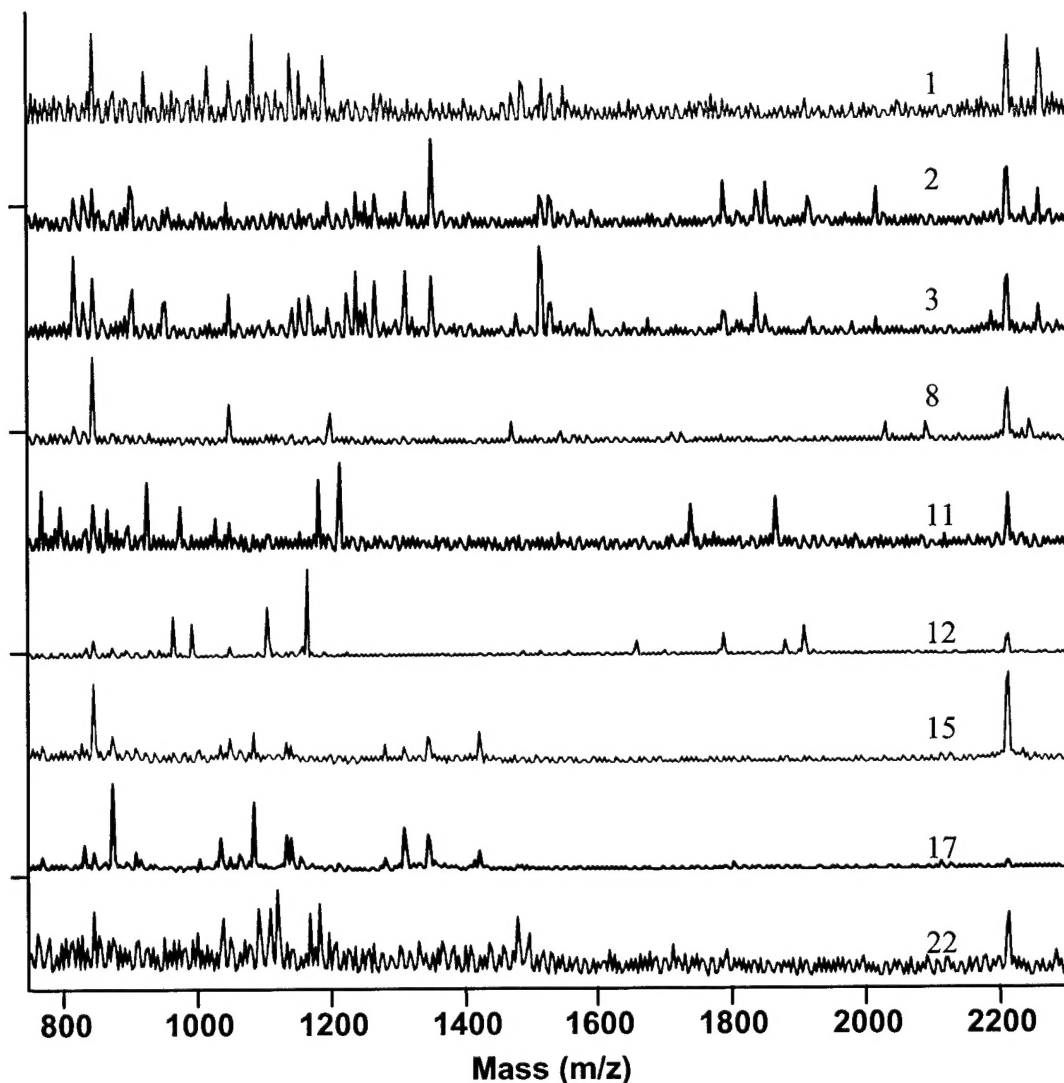
Figure 7. Representative MALDI TOF MS analyses of trypsin digests from various estradiol regulated and control proteins. Spectra were obtained on a Applied Biosystems Voyager DE in linear mode with alpha-cyano-4-hydroxy cinnamic acid matrix. Peptide mass profiles from these spectra were used to search the non-redundant protein database to identify the proteins. Calreticulin and Hsp90 were identified from spectra 1, and 2 and 3, respectively. Spectra 15 and 17 were obtained from the spot family shown in Figure 6D. Spectrum 22 corresponds to the downregulated protein shown in Figure 5A. Identities of additional proteins await further confirmatin by LC MSMS.

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A.

LLLEFTDTSYEEK
Calculated [M+H]$^+$ = 1588.75
Calculated [M+2H]$^{2+}$ = 794.88
Experimental [M+2H]$^{2+}$ = 793.80

B.

LATQSNEITIPVTFESR
Calculated [M+H]$^+$ = 1907.13
Calculated [M+2H]$^{2+}$ = 954.07
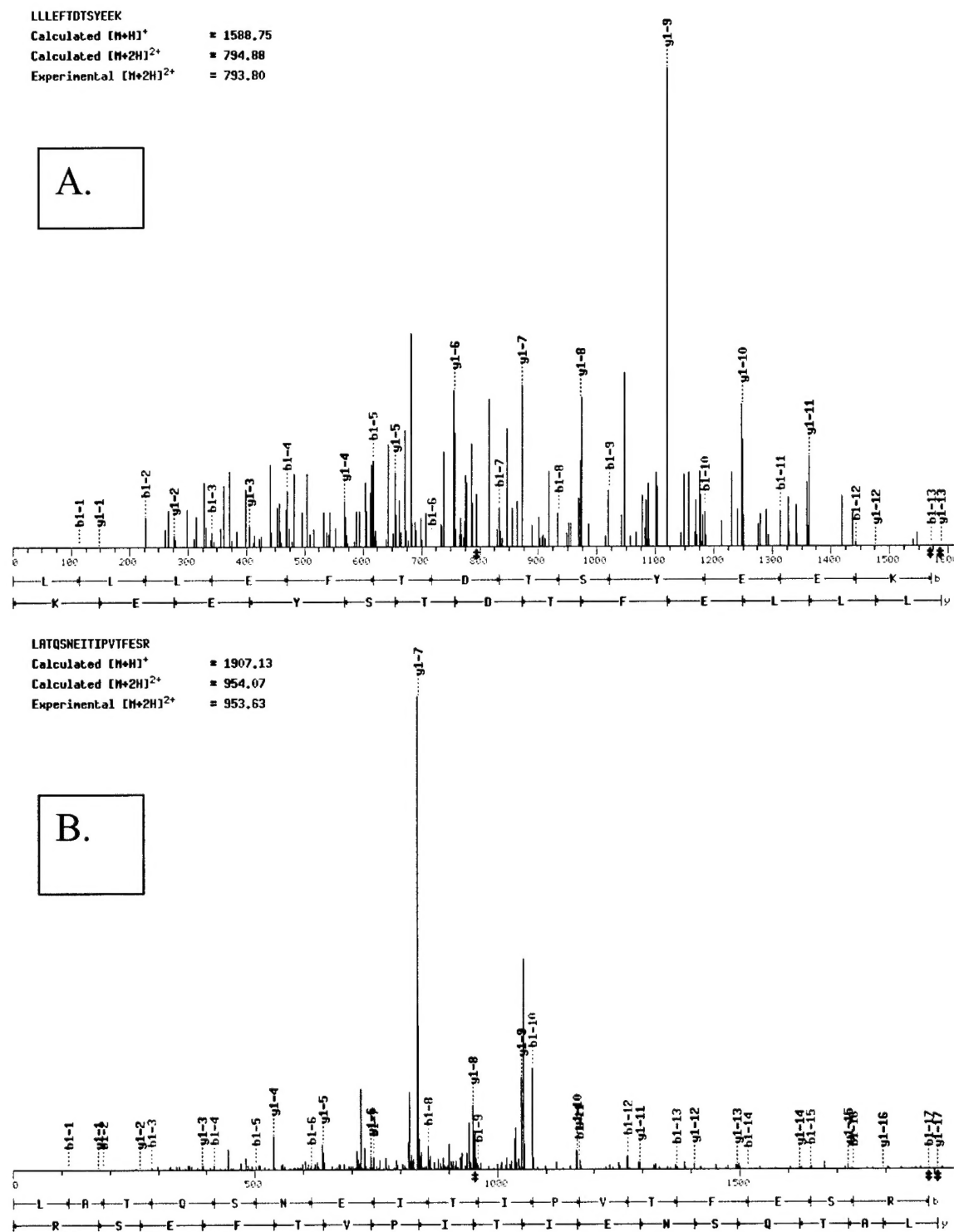Experimental [M+2H]$^{2+}$ = 953.63

Figure 8. Tandem mass spectra showing sequence assignments. Spectra were obtained on a LCQ quadrupole ion trap using nano-electrospray ionization while infusing trypsin digests of unknown proteins. Proteins were identified with the SEQUEST program search of the non-redundant protein database.